

## Research Article

# Clinical Evidence for the Importance of 1-Carbon Cycle Support in Subfertile Couples

Dominique Cornet<sup>1</sup>, Edouard Amar<sup>2</sup>, Marc Cohen<sup>3</sup> and Yves Ménézo<sup>4\*</sup>

<sup>1</sup>Clinique de la Muette, Rue Nicolo, 75016 Paris, France

<sup>2</sup>Cabinet Médical d'Andrologie, 17 avenue Victor Hugo, 75016 Paris, France

<sup>3</sup>Clinique Natecia, Avenue Rockefeller, Lyon France

<sup>4</sup>Laboratoire Clément, 17 avenue d'Eylau, 75016 Paris and London Fertility associates, 104 Harley street, London W1G7JD, UK

\*Corresponding author: Yves Ménézo, 4Laboratoire Clément, 17 avenue d'Eylau, 75016 Paris and London Fertility associates, 104 Harley street, London W1G7JD, UK

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## Abstract

This study assesses the effect of nutritional support directed towards the 1-C cycle on the fertility of male and female patients attending an ART program. In a first group, female partners of couples having failed at least one ART attempt were administered 1-C nutritional support prior to a further ART cycle. A second group comprised couples who had failed at least 2 assisted reproductive technology (ART) attempts, with male partners having elevated DNA fragmentation index (DFI) or nuclear decondensation index (SDI). The treatment consisted of compounds that play a major role in the 1-C cycle: B vitamins, chelated zinc and N-acetyl cysteine.

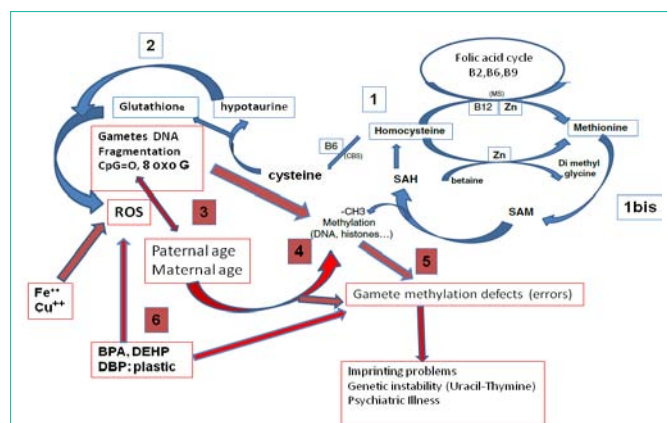
The first group of 100 treated female patients achieved a clinical pregnancy rate (PR) of 45%, with a high rate of spontaneous conception before ART. This was significantly higher ( $p=0.0001$ ) than the PR observed in the control group of 73 patients, with PR =13.7%. When the male partners alone (95) were treated, a significant decrease was observed for DFI and SDI vs. control (84), and this was associated with a significant increase in the delivery rates (47.4% vs. 21.4%,  $p=0.001$ ). Nutritional support of the 1-C cycle improves male and female fertility; this should not be overlooked, especially for women entering ART programs.

**Keywords:** ART; SDI; DFI; Oxidative stress; DNA

## Introduction

Oxidative stress (OS) represents a hazard to reproductive processes. The impact of OS on male fertility, *i.e.* on sperm quality, has been recognized since the pioneering works of Evenson et al. [1] in the early 1980's, and it now appears that damage caused by OS is shared equally between male and female [2]. Evidence indicates that damage can be attributed to roughly one-third of maternal origin, one-third paternal, and one-third shared between both partners. A better appreciation of sperm quality through estimation of DNA integrity is now available: fragmentation (DNA fragmentation index, DFI) and sperm nuclear decondensation index (SDI), or high DNA stainability (HDS). DFI and SDI correlate poorly with the classical WHO sperm parameters of count, motility and morphology, and without treatment are relatively stable over time [3-6]. The DFI increases considerably with age [3,5,6], but SDI decreases slightly, indicating an age-related weakened defense against reactive oxygen species [6]. Damage of paternal origin has multiple sources for ROS-linked decays that originate partly, but not entirely from the spermatozoon itself, *i.e.* the mitochondria [7]. More than 10 base oxidation products [8], DNA adducts [9], and DNA strand breaks [1], as well as some features of nuclear decondensation are ROS-derived. In the female partner, adduct formation and DNA base oxidation add to age-associated meiotic defects observed in oocytes from older patients; in combination with aberrant response to DNA damage and telomere recombination, all of these effects can lead to infertility or early miscarriage [10]. In theory, the oocyte/zygote can repair these chemical insults to some extent [8,11], but the capacity for repair is finite, and decreases with age [12]. When the oocytes' capacity for DNA repair is overwhelmed, a process of "tolerance" allows

mutations that lead to miscarriages and other later pathologies [13-14]. Recent observations in animals [15] have emphasized potential risks to postnatal development after performing micro-injection with highly fragmented DNA. It is tempting to try to reduce or avoid DNA damage related to ROS through consumption of anti-oxidants, and taking vitamins A, E and C, often coupled with selenium, seems an attractive proposition. Vitamins C and E can be either anti- or pro-oxidant. Equilibrium between oxidation and reduction status must be maintained over time. Vitamin C opens disulfide bridges of glutathione and protamines [16], leading to nuclear decondensation in the spermatozoon: oxidized glutathione is essential for padlocking and cross-linking protamines [17]. DNA decondensation induces chromosomal anomalies, and thus is deleterious for early development [18-19]. We recently observed [20] a process of sperm decondensation after treatment with mixtures of selenium and vitamins A, C, and E. The practice of ingesting large 'blind' doses of anti-oxidants is highly questionable [21], and has been shown to be poorly efficient, at least in women [22]: the final outcome, *i.e.* ongoing pregnancies and deliveries, are seldom taken into account. The fact that there is a strong correlation between oxidative stress and DNA methylation is no longer in question, and this has a further subsequent impact on transmitted diseases [23-25]. There is a clear biochemical explanation behind this concept, as seen in Figure 1. Oxidative stress has an influence on the one carbon (1-C) cycle, which is involved in homocysteine recycling with formation of methionine, necessary for DNA methylation. Through synthesis of cystine, products of the 1-C cycle include two major endogenous antioxidants: hypotaurine [26] present in the environment of oocytes, sperm and embryos, and glutathione. Ovarian stimulation has a negative impact on homocysteine metabolism [27]: homocysteine competes with the



**Figure 1:** Interrelations between oxidative stress and methylation processes  
 1- Correct recycling of Homocysteine allows generation of Cysteine and methionine which allows correct processes of methylation (1bis). 2- Correct generation of cysteine allows synthesis of Hypotaurine and glutathione, two potent inhibitors of ROS. 3- Generation of ROS induces DNA fragmentation. Advanced age decreases the ability to control ROS-linked decays. 4- High levels of homocysteine inhibit methylation processes in sperm physiology (methylation of the membrane lipids) and in sperm DNA. 5 - DNA methylation defects, whether or not linked to imprinting may result in negative transgenerational health problems. Unrepaired 8 oxoG leads to aberrant methylation at CpG sites, impair transcription and may affect telomere length. 6- Plastics Derived Endocrine Disruptors (BPA, DEHP and DBP) worsen all the negative steps.

same oocyte receptor for methionine, and thus inhibits methylation reactions that are necessary for imprinting [28]. The cysteine beta synthase (CBS) pathway allows recycling of homocysteine to some extent, but this pathway is not expressed in the human oocyte [29]. Therefore, until the time of genomic activation on the third day post-fertilization, the human zygote genome is poorly defended from the negative impact of homocysteine on supplies of methionine that are required to maintain methylation, with a downstream effect on imprinting. In addition, female and male reproductive physiology is now at risk from plastic-derived endocrine disruptors present at high levels in our current environment. This study was conducted in order to investigate the impact of nutritional support for the 1-C cycle in female and male partners consulting for ART.

**Materials and Methods**

All of the studies were performed at a French private clinic that is not required to have, and does not have, a reference Institutional Review Board; however the protocols were conducted in full conformity with the current revision of the Declaration of Helsinki. The protocol was carried out during the period January - August 2013, in an ART center performing 1800 IVF/ICSI cycles per year. All subjects gave their informed consent to participate in the study. Follow-up continued until the time of data collection in December 2013, and all pregnancies were then monitored and followed to their outcome.

**Female patients whose male partners had normal WHO parameters**

All of the female patients admitted to the program had primary infertility of at least 3 years’ duration, with a minimum of one previous failed ART treatment cycle (mean 2.0). All of the women were <43 years of age (as regulated by French law), and had no pregnancy with

**Table 1:** Sample characteristics of female patients treated vs control (Mann Whitney); Treated: 19 endometriosis (19%), 30 PCOS (30%); Control: 9 endometriosis (12.3%), 19 PCOS (27%).

	N (Patients)	FSH (SD)	AMH (SD)	Age (SD)	
				Maternal	Paternal
Treated	100	6.2 (1.7)	2.3 (1.5)	32.5 (4.1)	33.7 (4.0)
Control	73	7.9 (3.2)	2.4 (2.6)	34.7 (5.3)	36.8 (5.8)
p		0.02	0.12	NS	NS

**Table 2:** Supplement formulations (mG,% Guideline Daily Amounts)/ Procrelia Man™.

Compound	mg (%GDA)
N-acetyl cysteine	250
Zinc	15 (150%)
Vitamin E	10 (83.2%)
Vitamin B3	16.0 (100%)
Vitamin B6	1.4 (100%)
Vitamin B2	1,40 (100%)
Vitamin B9	0.4 (200%)
Vitamin B12	2.5x10 <sup>-3</sup> (100%)
Prickle pear extract	270
Betalaine (from beetroot)	40

Procrelia Women™ contains the same constituents + 40mG of Fish oil.

a previous partner. The male partners had sperm parameters above the threshold for normality, as determined by the “WHO laboratory manual for the examination and processing of human semen” (2010). Female partner serum FSH and AMH (anti-Mullerian hormone) levels were measured on Day 3 of the cycle, with no indication of ovarian failure in either the control or the study group (AMH >1.5µG/L and FSH <9 UI/L); FSH levels were slightly higher in the control group (7.9 vs 6.2, p=0.02) but AMH was lower in the treatment group (2.3 vs 2.42, p= 0.12, respectively). All values were considered to be in the normal range. The incidence of polycystic ovarian syndrome (PCOs) and endometriosis was close to 50% in both groups, with no statistical difference: these two pathologies are significantly linked to oxidative stress. Table 1 illustrates the background data obtained for the two groups.

Female patients in the treatment group received 2 tablets of Procrelia™ Woman (Parthenogen Lugano, CH, Nurilia, Lyon France) per day, starting three months before their ART protocol. As shown in Table 2, Procrelia™ Woman contains compounds of the one-carbon cycle, vitamins of group B, chelated Zn, N-acetyl cysteine, weak antioxidants and fish oil. To ensure activity, Zn must be added in a chelated form (www.efsa.europa.eu/, European Food Safety authority: EFSA-Q-2005-035, EFSA-Q-2005-133, EFSA-Q-2005-034, EFSA-Q-2005-038, EFSA-Q-2005-166, EFSA-Q-2005-033, EFSA-Q-2005-132, EFSA-Q-2005-036, EFSA-Q-2005-130).

**Male patients with abnormal (elevated) SDI and subnormal DFI whose female partner had no detected anomalies**

The duration of primary infertility in these couples was at least 3 years, with at least two failed ART (IVF/ ICSI) attempt, and no pregnancy with a previous partner. Oligoasthenospermic patients were excluded. Total sperm count and viability (eosin-nigrosin on at

**Table 3:** Pregnancies and deliveries: women treated only, male partner “normal”, treatment vs. Control.

	N (patients)	Pregnancies	Miscarriages	Deliveries (%)
Treated	100	45 <sup>1</sup> (45%)	5 (11%)	40 (40%)
Control	73	10 <sup>2</sup> (13.7%)	2 (20%)	8 (10.9%)
p		0.0001		

Statistics: Chi square;

\*1: Distribution of the pregnancies: 4/16 IVF (In vitro fertilization), 11/17 ICSI(Intra- cytoplasmic sperm injection), 30 spontaneous. No Artificial insemination with Husband (AIH)

\*2: Distribution of the pregnancies: 3/9 IVFs, 3/51 AIHs, 4/28 ICSIs, 0/9 frozen embryo transfers

**Table 4:** DFI and SDI before and after the treatment, only male partners treated, female partner “normal”, treatment vs controls.

	N	Age	DFI			SDI				
			Before	After	Delta	Before	After	Delta		
Treated	95	34.7 (4.31)	27 (6.8)	0.001	17.3	-9.7 (6.3)	26.9 (5.9)	0.032	18.9 (5.0)	-8.0
Control	83	37.8 (6.7)	26.9 (4.5)	0.32	25.9 (4.4)	1	30.5 (4.3)	0.15	31.1 (4.5)	0.6

Statistics: Chi square;

\*1: 4/16 IVF ( In vitro fertilization), 11/17 ICSI(Intra- cytoplasmic sperm injection), 30 spontaneous. No Artificial insemination with Husband (AIH).

\*2: 3/9 IVFs, 3/51 AIHs, 4/28 ICSIs, 0/9 frozen embryo transfers.

least 200 cells) in the ejaculates were performed according to WHO (2010) guidelines in an accredited laboratory (ISO 15189). All total counts in the ejaculates were above the WHO threshold for infertility: treated, 48.7 million/ml (SD: 43.8); control, 46.8 (29.7). Motility was greater than 15%: treated patients, 21.1 % (SD: 6.2); control, 17.6 (SD: 6.4), NS. None of the female partners had detectable pathologies: normal ovulatory profile, endocrinological parameters (FSH< 8UI/L, estradiol<40µg/L) and ovarian reserve (AMH>2µg/l). The female partners were <43 yrs of age, as recommended by the French Biomedicine Agency (moreover, patients > 43 yrs are not reimbursed by the Social security). This pilot study was not randomized with a placebo group; the control group consisted entirely of male patients who received no complementary treatment during their ART cycles. The male partners were under consultation for primary infertility (duration >3 yrs) with previous IVF/ICSI attempts, and no pregnancies with a previous partner. All had been tested for testicular volume and varicocele by testicular palpation and sonography, and testosterone levels were in the normal range. Sperm DFI was measured via flow cytometry terminal deoxynucleotidyl transferase UTP nick end labeling (Tunel) assay [30]. The SDI was measured by microscopy of 200 cells using aniline blue (AB) [31], since it has been shown that AB appears to be more specific than chromomycine [5] in detecting DNA fragmentation. Values for sperm DNA structure are presented in Table 3.

The patients took two tablets of Procrelia Man (™) per day for a period of 4 months. SDI and DFI were re-assessed after treatment. The control group received no supplement and was monitored after 20 wks. The composition of Procrelia Man is the same as Procrelia Woman, without fish oil extract (see Table 2).

SDI and DFI were measured at the end of the treatment period via blinded analysis. The choice of ART procedure was then decided post-consultation at the discretion of the gynecologist and the urologist.

**Statistics**

This investigation was carried out as a pilot study: the Mann–Whitney test (2 independent groups) was used for continuous

**Table 5:** Pregnancies and deliveries in treated and control group (male treatment with Procrelia Man) Female Partner normal.

	N	MATERNAL AGE	Pregnancies	Deliveries
Treated	95	32.9 (3.8)	49 (56%)* <sup>1</sup>	45 (47.4%)
			p= 0.001	
Control	84	34.6 (5.4)	23 (27.4%)* <sup>2</sup>	18 (21.4%)

\*1: 8 Spontaneous, 11/25 IVFs (44%), 12/30 ICSIs (40%), 11/20 AIHs (55%), 8/12 F/T embryo transfers (66%).

\*2 : 3/22 IVFs (13.6%), 13/29 ICSIs (44.8%), 6/15 AIHs (40%),1/18 F/T embryo transfers (5.5%).

variables. Basic descriptive statistics (mean, range and standard deviation) were determined as appropriate, and statistical analysis was performed using IBM SPSS statistical software. The Chi<sup>2</sup> test (test of homogeneity) was used to estimate the independence of two categorical variables, and for comparison of percentages.

**Results**

**Female patients whose male partners had normal WHO parameters (Table 3)**

The results show a highly significant increase in pregnancy rate for the treated group: 45% vs. 13.7% for the control group, p<0.001. The most significant observation is a very high spontaneous pregnancy rate prior to starting a proposed ART procedure in the treatment group: 30/100 per patients and 2/3rd of the pregnancies, vs. none in the control group. The number of miscarriages was insufficient for statistical analysis (11% vs. 20% in the control group).

**Male patients with abnormal (elevated) SDI and subnormal DFI whose female partner had no detected anomalies (Tables 4 and 5)**

Treatment modified sperm count and motility only marginally (count, +5.1%, control, +6.8%, NS); motility (+12%, control: -1.6%, p=0.01). As shown in Table 4, a significant decrease was observed for DFI (-36.3%, p=0.001) and SDI (-29.7%, p=0.32). The pregnancy rates were also significantly increased in the treated group: 56% vs. 27.4%, p = 0.001 (Table 5). The percentage of spontaneous pregnancies, before starting an ART attempt is also high in this group (8/95: 8.4% of the

patients and 8/49=16.3% of all of the pregnancies). It was impossible to determine an effect on miscarriage due to the small numbers (Treated: 8%, control: 21.7%).

## Discussion and Conclusions

The present study reports clinical pregnancy (CPR) and live birth rates (LBR) of 45% and 40% respectively in female patients treated with supplements to support the 1-C cycle. Of particular note is the fact that two-thirds (30/45) of the pregnancies were spontaneous, with conception occurring during the run-up to a planned ART cycle. This means that the oocytes have decreased their DNA damage and have also increased their DNA repair capacity. This perspective of the female contribution to a couple's infertility supports the observations published by Dattilo et al. [32]. When the female gamete is compromised, treating male partners alone with supplements involved in 1-C support leads to an immediate dramatic decrease in the LBR: this may indicate that improvements in sperm quality may be of little help in the presence of an added female factor. The same symmetrical observation can be made when males are treated; the CPR and LBR are increased (but to a lesser extent when compared to that observed for women). Although it is possible to determine the mechanisms behind the effects of treatment on sperm DNA structure, this is not the case in the female. As expected from published data [2] the female gamete is at risk from decays linked to oxidative stress. The 1-C cycle recycles homocysteine, and this represents a major effectors in regulation of female fertility. Homocysteine lies at the crossroads of the problem, especially in the case of the oocyte [27,33]. This feature is of particular concern in view of the fact that environmental conditions, especially plastic-derived endocrine disruptors (a) have estrogen-like compounds (see figure 1) and (b) are found in the urine of patients entering ART programs [34]. Ovarian stimulation increases in complexity with increasing levels of urinary BPA, associated with decreasing ovarian response; (c) Plastics Derived Endocrine Disruptors (BPA, DEHP and DBP) induce Epigenetic Transgenerational Inheritance in animals through an inhibitory effect on methylation processes, inducing epimutations (d) these compounds induce oxidative stress and mitochondrial dysfunction.

All of the oxidative stress markers are elevated in PCOs patients, who may also suffer effects on methylation via an increase in circulating homocysteine [35]; levels of circulating glutathione, (pivotal molecule in protection against ROS) are also decreased. This strongly suggests that oxidative stress may participate in the pathophysiology of PCOs and endometriosis [36], with a negative effect on oocyte quality and subsequently on embryo development and then on rates of miscarriage. Clearly, oxidative damage and methylation defects are relevant clinical targets that can be positively influenced by tailored nutritional supplementation: this is a feature that should not be overlooked in assisted reproduction technology, especially in consideration of the high rates of spontaneous conception revealed in this study. This type of nutritional support seems at least as important in women as in men. The negative impact of endocrine disruptors present in the environment with an effect on epigenetic reprogramming and reproduction is a further reason for positive consideration of these supplements. In the absence of therapeutic intervention, DFI and SDI are much more stable over time than are the classical semen parameters of count, motility and morphology

[4,5,6,37]. Our study confirms that antioxidant nutritional support resulted in a significant decrease in both DFI and SDI [30]. No variation was observed in the control group. This confirms that stimulating endogenous generation of antioxidants, *i.e.* glutathione and perhaps to a lesser extent hypotaurine, through the cystathionine beta synthase pathway (CBS) not only prevents DNA decays in terms of fragmentation, but also prevents nuclear decondensation. The protective antioxidant activity protects against oxidation of guanine, the most sensitive base. 8-oxo guanosine formation correlates with DNA fragmentation [31]. Telomeres, with repeats of TTAGGG, have a high G content. Critically, short telomeres are associated with sperm DNA fragmentation [38]. Telomere shortening induces defects in meiosis, fertilization and embryo development and may thus lead to infertility. Telomere dysfunction is one of the epicenters in reproductive aging [10].

The One-carbon cycle simultaneously allows homocysteine recycling to methionine and then S-adenosylmethionine (SAM), which is involved in epigenetic modifications necessary for further embryonic development. SAM is also involved in the synthesis of spermine and spermidine, both with a positive impact on DNA stability and chromatin-mediated regulation of gene expression. The effect of the 1-C cycle in sustaining methylation should not be overlooked: it is now common knowledge that sperm DNA methylation affects fertility, and further the quality of the conceptus through epigenetic mechanisms [39,40]. All of these observations reinforce the suggested link between reactive oxygen species (ROS) decays, paternal age, DNA methylation, some genetic alterations, psychic disorders in children [23, 29 40, 41] and in some cases carcinogenesis. One must not overlook the fact that ICSI is not a "miracle" techniques [42], and that upstream improvements can be made in ART techniques.

In conclusion, the present study demonstrates that nutritional support capable of activating and sustaining the 1-Carbon cycle is effective in improving the pregnancy outcome in couples consulting for infertility. It could also be considered as a form of protection against some of the potential risks for the children. None of these considerations should be overlooked, especially with regard to the increasing pollution by endocrine disruptors, true inhibitors of methylation, which induce epigenetic transgenerational inheritance of reproductive disease and sperm epimutations [43].

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